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A marked propensity for prostate cancer to arises in the peripheral prostate with aging. The Insulin-like Growth Factor-II (IGF-II) gene is an auto-paracrine growth stimulator that is an important positive modulator of cancer development. IGF-II typically demonstrates monoallelic, or imprinted, expression in adult tissues and indeed this pattern is maintained in the periurethral zone, a region where cancer development is rare. In addition, IGF-II loss of imprinting (LOI), as well as increased IGF-II expression, are common attributes of prostate cancer. It is our hypothesis to be tested that an age-dependent loss of IGF-II imprinting, resulting from age-dependent changes in DNA methylation, occurs specifically in the peripheral zone of the prostate and contributes to the increased risk for cancer development. To examine temporally when this loss of IGF-II imprinting occurs and the mechanisms underlying it we propose 3 Specific Aims: (1) To determine if IGF-II LOI in the peripheral prostate derives from stromal and/or epithelial cells; (2) To determine whether IGF-II LOI occurs as an age-dependent process in human prostate tissues that are uninvolved with cancer; and (3) To examine DNA methylation as a mechanism for any observed changes in the imprint status in prostate tissues. This proposal is significant and unique in testing whether regional epigenetic changes occur in histologically normal prostate tissues that are destined to become neoplastic. We expect to determine whether specific age-related, peripheral zone changes in methylation and imprinting occur in the general population and whether these changes are linked to prostate cancer development.

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## INTRODUCTION

Three important features of prostate cancer will be addressed in the present proposal that may provide a quantum leap in our understanding of the risk factors and development of prostate cancer. These features include: (1) A marked propensity for prostate cancer to arise in the peripheral prostate; (2) The multifocality of prostate cancer which implicates a generalized or field change in cancer susceptibility; and (3)

The important role of the Insulin-like Growth Factor (IGF) axis in both aging-related and genetic-related cancers. The Insulin-like Growth Factor-II (IGF-II) gene is a auto-paracrine growth stimulator that is an important positive modulator of cancer development. We will provide preliminary evidence that a loss of imprinting, or biallelic expression, of the IGF-II gene is an age-related specific epigenetic alteration that occurs in the peripheral prostate. IGF-II typically demonstrates monoallelic, or imprinted, expression in adult tissues and indeed this pattern is maintained in the periurethral zone, a region where cancer development is rare. In addition, IGF-II loss of imprinting (LOI), as well as increased IGF-II expression, are common attributes of prostate cancer. Since DNA methylation is the major determinant of gene imprinting, we would anticipate that a loss of IGF-II imprinting in the prostate will be associated with specific changes in the IGF-II/H19 promoter regions.

*It is our hypothesis to be tested that an age-dependent loss of IGF-II imprinting, resulting from age-dependent changes in DNA methylation, occurs specifically in the peripheral zone of the prostate and contributes to the increased risk for cancer development.*

To examine temporally when this loss of IGF-II imprinting occurs and the mechanisms underlying it we propose 3 Specific Aims: (1) To determine if IGF-II LOI in the peripheral prostate derives from stromal and/or epithelial cells; (2) To determine whether IGF-II LOI occurs as an age-dependent process in human prostate tissues that are uninvolved with cancer; and (3) To examine DNA methylation as a mechanism for any observed changes in the imprint status in prostate tissues. We propose to confirm our preliminary observations through a comprehensive analysis of IGF-II LOI and DNA methylation analysis in aging prostate tissues associated with and without cancer. These studies will utilize a unique tissue bank containing normal prostate tissues of various ages. Several innovative techniques including laser capture microdissection and quantitative allele-specific imprinting assays will also be employed.

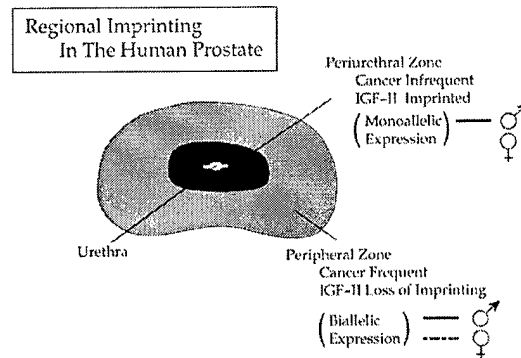


Figure 1: The IGF-II gene demonstrates monoallelic (paternal only) expression in the periurethral zone where benign prostatic hyperplasia originates. This pattern is found in most adult tissues. In the peripheral zone, where prostate cancer arises, expression is from both inherited alleles(4).

## BODY

**Task 1: To determine whether a LOI in IGF-II arises from prostate epithelial cells, stromal cells or both in normal human prostate tissues.**

1. *Acquisition and histologic analysis of prostate specimens, DNA production (Months 1-3):* During the interval of this grant we have histologically examined to exclude concomitant cancer, made DNA and screened approximately 52 samples for the *Apal* polymorphism. Twenty-one have been found to contain the *Apal* polymorphism. DNA has been extracted from these tissues.
2. *Laser capture microdissection of stromal and epithelial cells from normal peripheral zone prostate tissues and RNA isolation (Months 1-12):* We have utilized laser capture to separate the stroma from the epithelium in 5 tissues from the peripheral prostate of samples from men in their 60's. Adequate signal was obtained from 3 samples to date.
3. *Imprinting analyses using RT-PCR/restriction enzyme digestion and development of quantitative allele-specific PCR assay (Months 2-14):* Our efforts have focussed on i) improving the quantitative aspects of the imprinting assay and ii) assessing whether biallelic expression occurs in the stroma or epithelia. Using our Biorad iCycler iQ™ quantitative PCR machine we examined a quantitative assay for determining the allele-specific expression of *IGF-II*. We designed two specific TaqMan™ probes labelled with different fluorophores (FAM and VIC) that anneal specifically to either one or the other polymorphism (T or C) in exon 9 of the *IGF-II* gene(1). These probes contain quenchers. The probes are displaced during primer extension by Taq polymerase resulting in detection of the now unbound fluorophore. This assay demonstrates linear values when known ratios of the *IGF-II* polymorphism are compared. However, we have found that with the FAM probe as designed, it was relatively insensitive in detecting lower levels of signal. Two additional probes were generated without success at improving the sensitivity of this assay. In part, difficulty in probe design is due to the high G:C ratio of this region of the gene. As an alternate method we began utilizing a published technique employing radiolabeled primers and single cycle amplification (2;3) that is more laborious however avoids the PCR bias associated with the conventional assay. We have found that in all 3 samples of normal peripheral prostate analyzed to date, both the epithelium and stroma demonstrate biallelic expression. **Figure 2** This is remarkable since the majority of

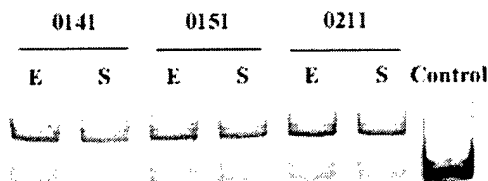


Figure 2.

tissues maintain a tight regulation of this gene. The epithelial-specific change suggests a regional epigenetic cell-specific alteration may predispose these cells to the formation of cancer. This would support our hypothesis. Furthermore, this

validates the epithelium as the cell of origin for prostate cancer. The stromal expression may function through an inductive (paracrine) effect on the epithelium. Stromal-specific alterations are important in the induction of epithelial cancers and could possibly represent an important clonal change(4;5). These findings are reproducible with multiple assay techniques and runs.

4. Statistical analysis (Months 12-14): Additional samples will be analyzed to reach statistical significance.

**Task 2 : To examine the frequency of LOI in IGF-II in aging human prostate tissues containing no associated prostate cancer.**

1. *Acquisition of non-tumor associated peripheral zone prostate tissues from different ages and DNA production (Months 10-12):* We have assessed 42 separate specimens from men of various ages for the presence of the Apal polymorphism. To date, 12 samples have yielded informative samples. Additional tissue libraries will be screened.
2. *Microdissection and isolation of RNA (Months 12-24)*
3. *Imprinting analyses using RT-PCR/restriction enzyme digestion and development of quantitative allele-specific PCR assay and statistical analysis (Months 12-26)*

**Task 3: To examine whether methylation alterations underlie differences in IGF-II imprinting in the human prostate.**

As an approach to begin to identify areas of the IGF-II/H19 locus that are important in epithelial cell aging, we have utilized an *in vitro* model of human prostate epithelial cell senescence. Senescence is an *in vitro* model of aging that recapitulates many of the gene changes seen in *in vivo* aging(6). Utilizing collagen-coated plates and a low serum media to exclude fibroblasts, as we have described(7), human prostate epithelial cells are cultured through 30-40 population doublings before growth ceases and a characteristic senescent morphology and senescence-associated  $\beta$ -galactosidase staining(7) develops. . In all epithelial cultures, a complete relaxation of the *IGF-II* imprint was found with passage to senescence. One of the strongest candidates for control of the *IGF-II* imprint is alteration in DNA methylation(8;9). In addition, DNA methylation changes occur consistently in aging both *in vivo* and *in vitro*(10). These changes include both a global hypomethylation as well as regional hypermethylation of selected CpG islands, including the estrogen receptor in the human colon(11).

We analyzed the methylation status of four CpG islands within the IGF-II/H19 locus (termed methylated regions (MR)) that had demonstrated differential allelic methylation patterns in the mouse and/or human (12-15). DNA was harvested from proliferating and senescent cells and treated with sodium bisulfite in a reaction that converts unmethylated cytosines to thymidine, but methylated cytosines remain unaltered. After PCR, individual alleles were cloned and sequenced.

One differentially methylated region in the human, intergenic between H19 and IGF-II, appears to harbor a methylation imprinting mark for the IGF-II gene (16;17). Deletion or hypermethylation of this CpG island has been demonstrated in mouse models to lead to biallelic IGF-II expression (15;18). As seen in **Figure 3**, one allele is completely methylated at more than 25 CpG sites in MR3. We find that as 3 epithelial cell cultures were passaged to senescence, the other allele becomes obligatorily methylated at CpG sites 23-25 (containing a *HpaII* site), representing a gain of  $28\% \pm 7\%$  in methylated alleles. Complete methylation at these sites was also found in senescent cultures enriched for senescent cells by sorting based on increased side-scatter (Figure 3B).

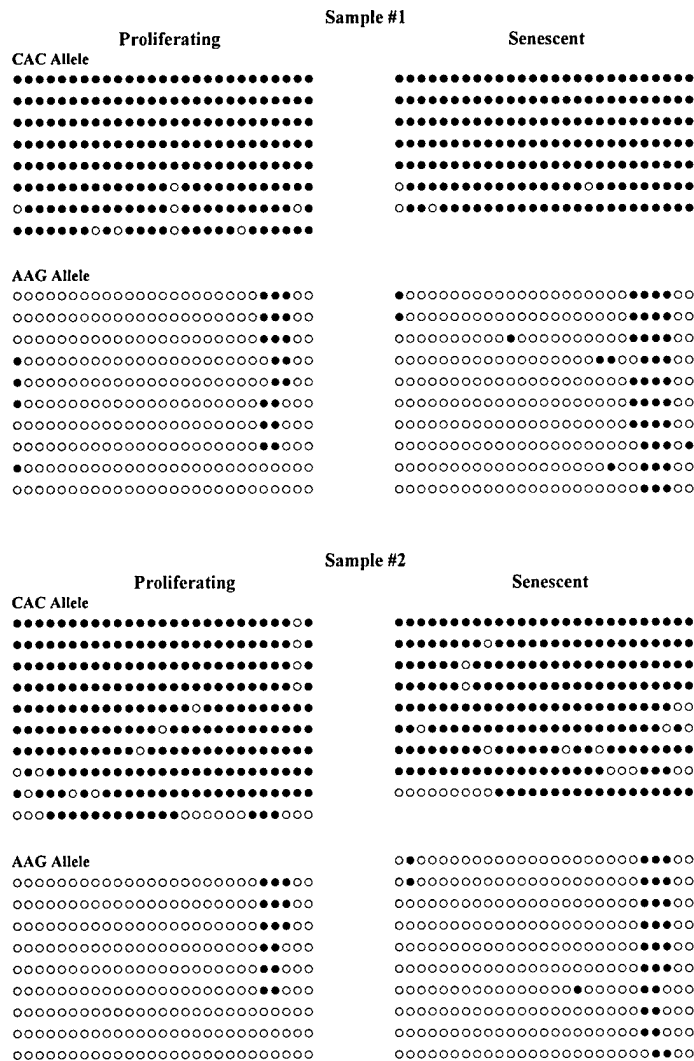


Figure 3.

A second region (MR2) located within exon 9 contains a CpG island that demonstrates methylation loss in mice with biallelic expression and has been reported to contain differential methylation in humans (19;20). We found that half of the alleles at this region did contain fewer methylated sites (<30% of total) than other alleles (>70%) suggesting some differential methylation exists between alleles in these human samples (data not shown). Analysis of the methylation status of individual alleles in this region demonstrated a 30% increase in methylation at senescence that occurred preferentially across 6 CpG sites (1-7) including one *HpaII* site. The binding sites for several nuclear proteins have been identified in MR2 that may be disrupted by hypermethylation (19). Analysis of 27 CG dinucleotides within exon 4 of the P2 promoter (MR1), containing a differentially methylated region in the mouse and a dense CpG island in the human (12), demonstrated no regional or globally significant changes in methylation (data not shown). Methylation levels did not vary between alleles at this CpG island indicating a differentially methylated region does not exist in human samples. Thus, consistent methylation changes occur in region MR3 and MR2 and these mark changes in epithelial cell aging in culture. These identified areas will be used to assess DNA samples from human tissues.

1. *Microdissection and isolation of DNA from imprinted and loss of imprinting prostate tissues (Months 24-32)*. As noted above 21 informative samples have been determined.
2. *Treatment of DNA with sodium bisulfite, PCR of CpG-enriched regions and sequencing (Months 24-34)*
3. *Quantitative methylation-sensitive single-primer extension analysis of specific sites (Months 24-36)*

#### **KEY RESEARCH ACCOMPLISHMENTS**

- We have demonstrated that both the epithelium and the stroma express the IGF-II gene from both alleles.
- We have analyzed a series of CpG islands in aging epithelial cells *in vitro* and have identified several loci that alter methylation status with increased population doublings.

#### **REPORTABLE OUTCOMES**

Presentations: 2nd International Conference on Prostate Cancer Research, Iowa City, Iowa. October 2002. "Senescence In Human Epithelial Cells Is Characterized by a Loss Of IGF-II Imprinting" Vivian X. Fu<sup>1</sup>, Steven R. Schwarze<sup>1</sup>, Mara Feld<sup>1,2</sup>, Lisa Grabert<sup>1</sup>, John Svaren<sup>3</sup>, and David F. Jarrard<sup>1</sup>

Funding: An RO1 (CA97131) was successfully funded this summer based in part on data obtained from this grant looking at age related changes in imprinting in the mouse prostate and cancer susceptibility.



## CONCLUSIONS

Both stroma and epithelia from peripheral prostate tissue in older men expression biallelic expression of IGF-II. This is in contrast to the tight regulation in other tissues. This finding may be important in the susceptibility of the prostate for developing age-related cancers.

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